

## **EXHIBIT 5**

# Five Different Anti-Prostate-specific Membrane Antigen (PSMA) Antibodies Confirm PSMA Expression in Tumor-associated Neovasculature<sup>1</sup>

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## ABSTRACT

Prostate-specific membrane antigen (PSMA) is a type II integral membrane glycoprotein that was initially characterized by the monoclonal antibody (mAb) 7E11. PSMA is highly expressed in prostate secretory-acinar epithelium and prostate cancer as well as in several extraprostatic tissues. Recent evidence suggests that PSMA is also expressed in tumor-associated neovasculature. We examined the immunohistochemical characteristics of 7E11 and those of four recently developed anti-PSMA mAbs (J591, J415, and Hybritech PEQ226.5 and PM2J004.5), each of which binds a distinct epitope of PSMA. Using the streptavidin-biotin method, we evaluated these mAbs in viable prostate cancer cell lines and various fresh-frozen benign and malignant tissue specimens. In the latter, we compared the localization of the anti-PSMA mAbs to that of the anti-endothelial cell mAb CD34. With rare exceptions, all five anti-PSMA mAbs reacted strongly with the neovasculature of a wide spectrum of malignant neoplasms: conventional (clear cell) renal carcinoma (11 of 11 cases), transitional cell carcinoma of the urinary bladder (6 of 6 cases), testicular embryonal carcinoma (1 of 1 case), colonic adenocarcinoma (5 of 5 cases), neuroendocrine carcinoma (5 of 5 cases), glioblastoma multiforme (1 of 1 case), malignant melanoma (5 of 5 cases), pancreatic duct carcinoma (4 of 4 cases), non-small cell lung carcinoma (5 of 5 cases), soft tissue sarcoma (5 of 6 cases), breast carcinoma (5 of 6 cases), and prostatic adenocarcinoma (2 of 12 cases). Localization of the anti-PSMA mAbs to tumor-associated neovasculature was confirmed by CD34 immunohistochemistry in sequential tissue sections. Normal vascular endothelium in non-cancer-bearing tissue was consistently PSMA negative. The anti-PSMA mAbs reacted with the neoplastic cells of prostatic adenocarcinoma (12 of 12 cases) but not with the neoplastic cells of any other tumor type, including those of benign and malignant vascular tumors (0 of 3 hemangiomas, 0 of 1 hemangioendothelioma, and 0 of 1 angiosarcoma). The mAbs to the extracellular PSMA domain (J591, J415, and Hybritech PEQ226.5) bound viable prostate cancer cells (LNCaP and PC3-PIP), whereas the mAbs to the intracellular domain (7E11 and Hybritech PM2J004.5) did not. All five anti-PSMA mAbs reacted with fresh-frozen benign prostate secretory-acinar epithelium (28 of 28 cases), duodenal columnar (brush border) epithelium (11 of 11 cases), proximal renal tubular epithelium (5 of 5 cases), colonic ganglion cells (1 of 12 cases), and benign breast epithelium (8 of 8 cases). A subset of skeletal muscle cells was positive with 7E11 (7 of 7 cases) and negative with the other four anti-PSMA mAbs. PSMA was consistently expressed in the neovasculature of a wide variety of malignant neoplasms and may be an effective target for mAb-based antineovasculature therapy.

## INTRODUCTION

PSMA<sup>3</sup> is a type II membrane glycoprotein of  $M_r \sim 100,000$  that was initially characterized by the mAb 7E11 (1, 2). Recent studies have confirmed the location of the PSMA gene on chromosome 11p and have demonstrated the existence of a related PSMA-like gene on 11q (3-5). Two variant forms of PSMA, initially predicted to exist as PSMA, and a spliced form, PSM', have been subsequently confirmed. PSMA is highly expressed in benign prostate secretory-acinar epithelium, prostatic intraepithelial neoplasia, and prostatic adenocarcinoma (2, 6-8), and evidence suggests that PSMA expression is greatest in high-grade and hormone-insensitive cancers (2, 9-11). A shorter, alternatively spliced and presumably cytosolic form of PSMA, named PSM', is the predominant form expressed in benign prostate epithelium (12, 13). Several studies have shown that anti-PSMA mAbs bind to several nonprostate tissues, including duodenum and kidney (6, 14, 15), and to the vasculature associated with solid malignant tumors (15, 16).

The function of PSMA is currently under investigation. Pinto *et al.* (17) demonstrated that PSMA has a folate hydrolase-type of activity because LNCaP cells were shown to hydrolyze  $\gamma$ -glutamyl linkages in methotrexate triglutamate. Others have demonstrated that PSMA has a neuropeptidase-type function (18, 19). On the basis of these enzymatic characteristics, the nomenclature committee of the International Union of Biochemistry and Molecular Biology has recommended for PSMA the formal name of glutamate carboxypeptidase (EC 3.4.17.21; Ref. 20).

The 7E11 antibody is a specific murine IgG mAb that was derived after immunization of mice with preparations from the LNCaP human prostate cancer cell line (1). 7E11 has been well characterized and is known to bind an intracellular epitope of PSMA not present on PSM'. As a result, 7E11 does not bind viable prostate cancer cells (1, 16, 21). Modified by the addition of <sup>111</sup>In, 7E11 is used currently at some centers as an imaging agent *in vivo*. Clinical trials have demonstrated that this radioimmunoconjugate of 7E11, known as <sup>111</sup>In-capromab pendetide, may be a useful adjunct in identifying and localizing metastatic or recurrent prostate cancer (22-25).

A number of other anti-PSMA mAbs have been developed recently that bind epitopes that are distinct from that recognized by 7E11 (13, 16). For example, the mAbs J591, J415, J533, and E99 bind to the extracellular PSMA domain (16). Investigators at Hybritech Inc. (San Diego, CA) have identified and purified the mAb PEQ226.5, which binds the peptide backbone of the PSMA extracellular domain. In addition, investigators at Hybritech Inc. have identified PM2J004.5, which binds an epitope of the intracellular PSMA domain that is distinct from that bound by 7E11 (13).

The purpose of this study was to compare the immunohistochemical profiles of four recently developed anti-PSMA mAb to that of 7E11. Specifically, we evaluated these mAbs in prostate cancer cell lines, benign and malignant prostate tissue, benign nonprostate tissue,

<sup>3</sup> The abbreviations used are: PSMA, prostate-specific membrane antigen; mAb, monoclonal antibody; OC, organ-confined.

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and a variety of malignant tissues. In the latter, we sought further to confirm PSMA expression in tumor-associated neovasculture.

## MATERIALS AND METHODS

**Tissue Specimens and Antibodies.** The LNCaP, PC3, and PC3-PIP (PC3 cells transfected with PSMA<sup>4</sup>) were obtained from cell lines cultured in the George M. O'Brien Urology Research Center at Memorial Sloan-Kettering Cancer Center. Fresh-frozen tissue samples from male and female patients were randomly obtained from the Memorial Sloan-Kettering Cancer Center institutional tissue bank. Twenty different benign tissue types, including prostate tissue, were examined, as were the following tumor types: conventional (clear cell) renal cell carcinomas, transitional cell carcinomas of the urinary bladder, testicular-embryonal carcinoma, colonic adenocarcinomas, neuroendocrine carcinomas, glioblastoma multiforme, malignant melanomas, pancreatic duct carcinomas, non-small cell lung carcinomas, soft tissue sarcomas, benign and malignant vascular tumors, breast carcinomas, and prostatic adenocarcinomas. The 7E11 mAb was provided by Cytogen, Inc. (Princeton, NJ). The J591 and J415 antibodies were recently developed, and their characteristics were demonstrated previously (16). The mAbs PEQ226.5 and PM2J004.5 were provided by Hybritech Inc. (San Diego, CA) and also described previously (13). The anti-endothelial cell mAb CD34 (Immunotech, Coulter Company, Opa Locka, FL) was used for comparative immunohistochemical reactions in all cancerous tissue types.

**Immunohistochemistry.** LNCaP, PC3, and PC3-PIP were grown in cell culture wells to ~80% confluence. Immunohistochemical studies were then performed on the different cell types in either a viable or a fixed state. For fixation, the cells were treated with 10% buffered formalin for 10 min. The cells were then incubated with the different mAbs at 5 µg/ml at room temperature for 45 min. For live cells, after incubation with the primary antibody under the same conditions, the cells were then fixed in cold 10% buffered formalin for 10 min. The immunohistochemical reaction was completed by the streptavidin-biotin method. Briefly, the sections were washed thoroughly in 1.0% PBS, and biotinylated secondary antibody, horse anti-mouse IgG, was added for 60 min. After washing with PBS, streptavidin was added to the specimens for 60 min, and the slides were washed again in PBS. Next, the specimens were immersed for 5 min in a fresh solution of 0.06% diaminobenzidine tetrachloride and 0.01% hydrogen peroxide. Following washing, the sections were counterstained with hematoxylin, dehydrated, and mounted.

Tissue samples were snap-frozen in OCT compound placed in isopentane and stored at -70°C. Multiple 5-µm cryostat tissue sections were then cut and fixed in cold acetone (4°C) for 12 min. Prior to primary mAb incubation, the specimens underwent 30-min incubation with a normal horse blocking serum 1:20 in 2.0% BSA. The primary antibody incubations (5 µg/ml) were then performed with 7E11, J591, J415, PEQ226.5, PM2J004.5, and CD34 (in the cancer cases) for 60 min at room temperature. The remainder of the immunohistochemical reaction was completed using the streptavidin-biotin method as described previously. In tissue with known significant quantities of endogenous biotin, the immunoperoxidase method was used with rabbit antimouse immunoglobulin-peroxidase as the secondary antibody (Envision; DAKO Corp., Carpinteria, CA). In all tissue sections, negative controls were performed using blocking serum in place of the primary antibody. The immunohistochemical reactivities of all of the mAbs were then evaluated and compared.

## RESULTS

**Tumor-associated Neovasculture.** With rare exceptions, all five anti-PSMA mAbs bound tumor-associated neovasculture of nonprostatic tumors (Table 1 and Fig. 1). The neovasculture of one breast carcinoma and one soft tissue sarcoma (myxofibrosarcoma) showed no immunoreactivity; however, both contained CD34-positive vasculature. The four cases of breast carcinoma with PSMA-positive neovasculture were ductal carcinomas, and the one PSMA-negative case

Table 1 Results of PSMA immunohistochemistry in tumor cells and tumor-associated neovasculture

Tumor	No. of positive tumors/total no. of tumors studied	
	Tumor cells	Neovasculture
Conventional renal cell carcinoma	0/11	11/11
Transitional cell carcinoma	0/6	6/6
Testicular embryonal carcinoma	0/1	1/1
Colonic adenocarcinoma	0/5	5/5
Neuroendocrine carcinoma	0/5	5/5
Glioblastoma multiforme	0/1	1/1
Malignant melanoma	0/5	5/5
Pancreatic duct carcinoma	0/4	4/4
Non-small cell lung carcinoma	0/5	5/5
Soft tissue sarcoma	0/6	5/6
Breast carcinoma	0/6	5/6
Hemangioma	0/3	0/3
Hemangioperithelioma	0/1	0/1
Angiosarcoma	0/1	0/1
Angiolipoma	0/1	0/1
Angiomyolipoma	0/2	0/2
Prostatic adenocarcinoma	12/12	2/12

was lobular carcinoma. Interestingly, only a small subset of prostate cancer specimens showed PSMA-positive neovasculture (2 of 12 cases). In these cases, we found the CD34-stained sections to be useful in localizing so-called "hot spots" of neovasculture that we then compared to the anti-PSMA mAb-stained sections. This helped us confirm the location of vessels amid strongly PSMA-positive tumor cells. We noted no significant histological differences between prostate cancers with PSMA-positive neovasculture and those with PSMA-negative neovasculture. In all of the tumors, 7E11, J591, J415, PEQ226.5, and PM2J004.5 mAbs bound neovasculture in a like manner (Fig. 2). The results of CD34 immunohistochemistry in sequential tissue sections confirmed localization of the anti-PSMA mAbs to neovasculture endothelium (Fig. 2). In contrast to tumor-associated neovasculture, none of the anti-PSMA mAbs reacted with vasculature in the non-cancer-bearing tissue sections. The staining intensity of the external domain-binding mAbs (J591, J415, and PEQ226.5) in tumor-associated neovasculture was greater than that of the internal domain-binding mAbs (7E11 and PM2J004.5).

**Malignant Tumor Cells.** All 12 prostate cancer cases were strongly PSMA positive, and all nonprostate tumor cells were PSMA negative (Table 1). All vascular tumors were CD34 positive but PSMA negative.

**Prostate Cancer Cell Lines.** The external domain-binding mAbs (J591, J415, and PEQ226.5) bound viable LNCaP and PC3-PIP cells that are known to express PSMA. In contrast, the internal domain-binding mAbs (7E11 and PM2J004.5) did not bind viable LNCaP and PC3-PIP cells (Fig. 3). After formalin fixation, all anti-PSMA mAbs, including 7E11 and PM2J004.5, reacted with LNCaP and PC3-PIP cells. None of the mAbs bound viable or formalin-fixed PC3 cells that are known to lack PSMA expression.

**Benign Tissues.** Although benign prostatic secretory-acinar epithelium displayed heterogeneous staining with the five mAbs, all 28 benign prostate cases were PSMA positive. Immunoreactivity was typically concentrated at the luminal aspect of the cytoplasmic membrane. Basal epithelium and stromal cells were PSMA negative. The immunoreactivity of the benign secretory-acinar epithelium was less intense than that of prostatic adenocarcinoma, and the staining intensity of the external domain-binding mAbs J591, J415, and PEQ226.5 was greater than that of the internal domain-binding mAbs 7E11 and PM2J004.5 (data not shown).

The anti-PSMA mAbs reacted with several of the 19 benign non-prostate tissues (Table 2). All five mAbs reacted with duodenal

<sup>4</sup> J. B. Latouche and M. Sadelain, unpublished observations.

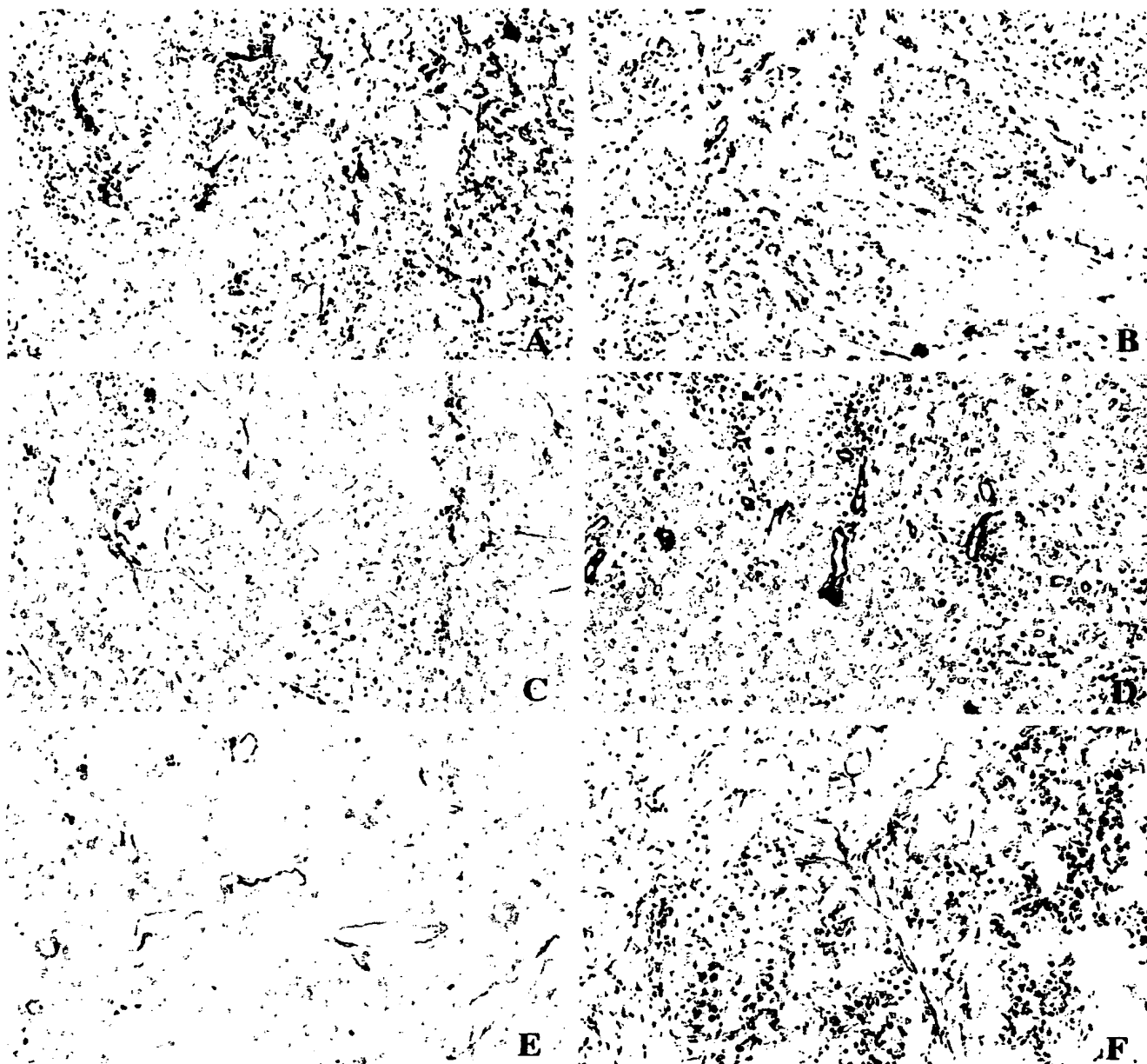


Fig. 1. PSMA expression in tumor-associated neovascularity. Immunohistochemical reactivity with external domain-binding anti-PSMA mAbs J591 or PEQ226.5 in representative cancer types. A, J591, breast cancer; B, PEQ226.5, transitional cell carcinoma of the urinary bladder; C, J591, malignant melanoma; D, PEQ226.5, non-small cell lung carcinoma; E, J591, soft tissue sarcoma; and F, J591, neuroendocrine carcinoma.

columnar (brush border) epithelium (11 of 11 cases), renal proximal tubular epithelium (5 of 5 cases), benign breast epithelium (8 of 8 cases), and colonic ganglion cells (1 of 12 cases). In skeletal muscle, a subset of muscle fibers were positive only with 7E11 and negative with the other four mAbs (Fig. 4). The vasculature in all benign tissues was uniformly PSMA negative. The staining intensity of these PSMA-positive benign tissues was less than that of prostate cancer and tumor-associated neovascularity.

## DISCUSSION

Our study confirms PSMA expression in the neovascularity of a wide spectrum of malignant neoplasms. Specifically, we found PSMA expression in various epithelial tumors (carcinomas), neuroendocrine tumors, and mesenchymal tumors (soft tissue sarcomas) and in ma-

lignant melanoma and glioma. In contrast to previous studies, we used five anti-PSMA mAbs, each of which binds a different epitope of the intracellular or extracellular PSMA domain. Thus, our results provide further evidence that PSMA, rather than a PSMA-like molecule, is expressed in tumor-associated neovascularity. Also in contrast to previous studies, we confirmed localization of PSMA to endothelial cells with the mAb CD34, an anti-endothelial cell marker used to study angiogenesis and determine microvessel density (26–30).

Our findings are consistent with previous studies showing PSMA expression in tumor-associated neovascularity. For example, Silver *et al.* (15) demonstrated 7E11 binding and “neoexpression of PSMA in endothelial cells” in a subset of tumors, including renal cell carcinoma (unspecified type), transitional cell carcinoma of the urinary bladder, and colonic adenocarcinoma. More recently, Liu *et al.* (16) studied

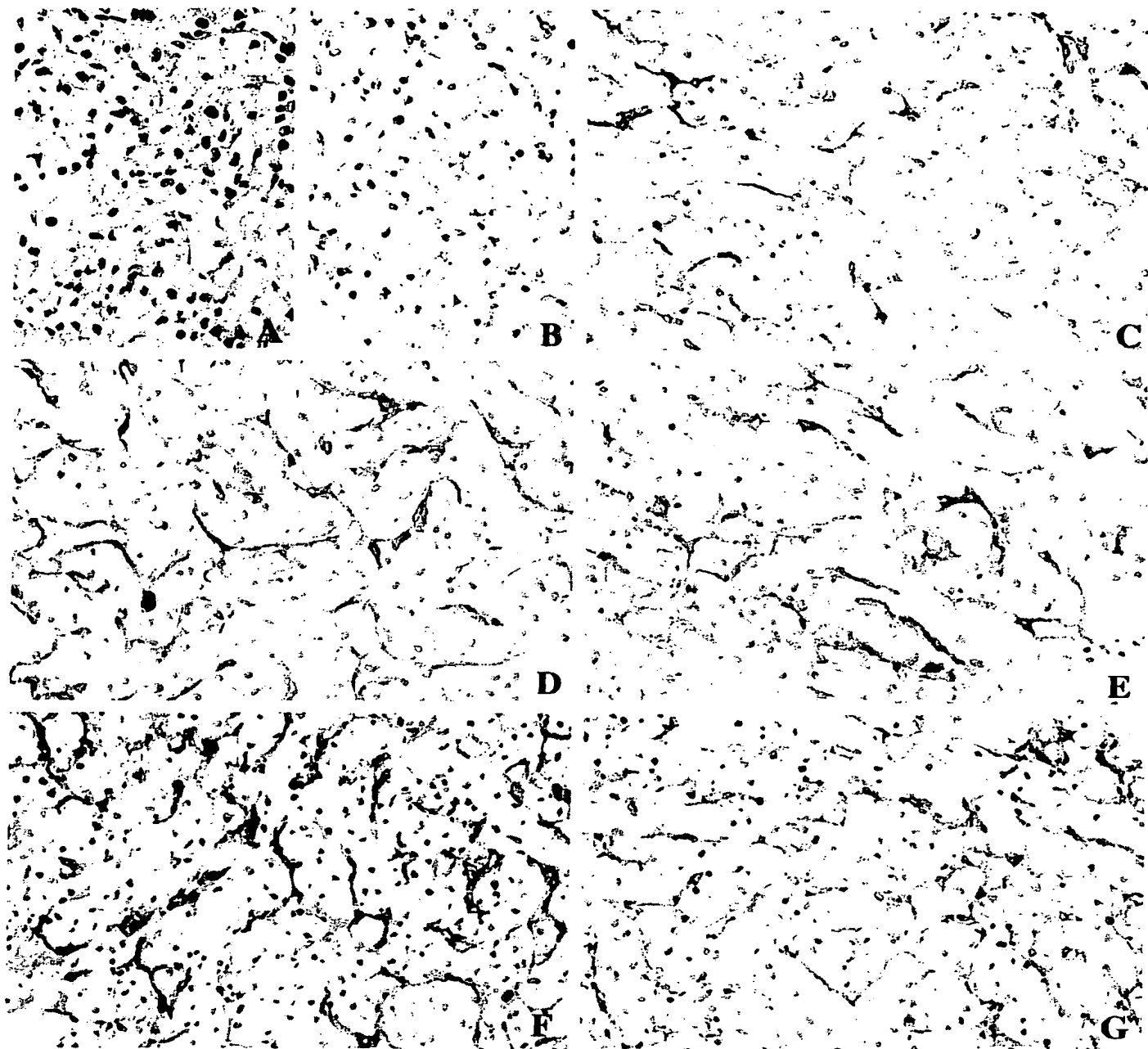


Fig. 2. Binding profile of the five anti-PSMA mAbs and CD34 in the neovascularity of conventional (clear cell) renal cell carcinoma. A, H&E-stained section; B, CD34; C, 7E11; D, J591; E, J415; F, PEQ226.5; and G, PM2J004.5.

four external domain-binding anti-PSMA mAbs (J591, J415, J533, and E99) and showed that each bound the tumor-associated neovascularity in several nonprostatic carcinomas. Although it is unclear whether PSMA is produced by endothelial cells of tumor-associated neovascularity or whether it is produced in other tissues and sequestered from the serum, we favor the former because PSMA is expressed only in a limited number of benign tissues and in prostate cancer but is not expressed in other malignant cell type. In addition, circulating PSMA has not been demonstrated in serum.<sup>5</sup> Additional studies, however, are necessary to confirm this hypothesis.

We found that endothelial cell expression of PSMA was restricted

to the neovascularity of malignant neoplasms. In fact, neither the vascular endothelial cells of benign tissues nor the neoplastic cells of vascular tumors expressed PSMA. These results suggest that endothelial cell-PSMA expression may be stimulated by one or more tumor-secreted angiogenic factors. The fact that all of the vascular neoplasms we studied, including the one example of angiosarcoma, were PSMA negative is not surprising, given that, in these tumors, the endothelium itself is neoplastic and, presumably, not stimulated by angiogenic factors. The presence or absence of PSMA expression in benign neovascularity (*e.g.*, granulation tissue, endometrium, and so on) remains to be established.

The neovascularity associated with OC prostatic adenocarcinoma only rarely expressed PSMA. Others also have found no detectable

<sup>5</sup> H. Liu and N. H. Bander, unpublished observations.

A



B

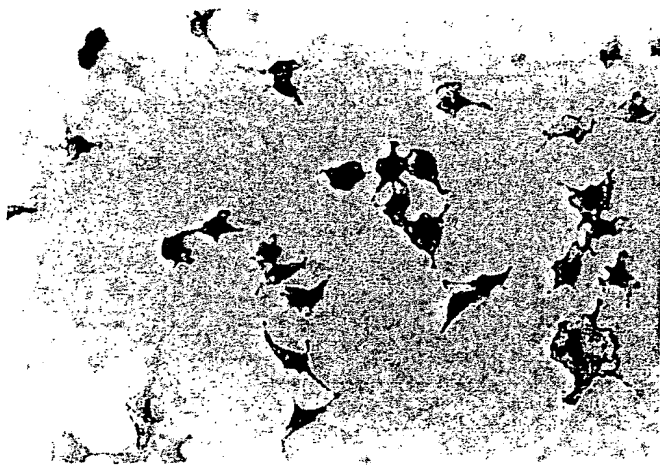


Fig. 3. Comparative immunohistochemistry in viable PSMA-expressing PC3-PIP cells. A, 7E11 demonstrating no immunoreactivity. B, J591 demonstrating positive immunoreactivity with live cells.

PSMA expression in OC prostate cancer-associated neovascularity (9, 15). These observations are remarkable given the ubiquity of PSMA expression in tumor-associated neovascularity of other cancer types. They are, however, not altogether surprising, given the histological features of OC prostate cancer. For example, in contrast to many other epithelial tumors such as ductal carcinoma of the breast or pancreas, OC prostate cancer typically is not associated with an exuberant host-stromal reaction. Lobular carcinoma of the breast, like prostatic adenocarcinoma, typically does not induce a marked desmoplastic stromal response. Interestingly, the one breast cancer specimen in our series with PSMA-negative neovascularity was an example of lobular carcinoma. These results suggest that PSMA expression in tumor-associated neovascularity may be related to the degree and nature of neoangiogenesis. The relationship between primary tumor stage in different malignancies and PSMA expression in neovascularity is unknown.

Consistent with most previous studies, we found that mAbs to the intracellular PSMA domain (7E11 and PM2J004.5) do not bind viable prostate cancer cells, whereas mAbs to the external domain (J591, J415, and PEQ226.5) do bind live cells (16, 21). Only one study has reported 7E11 binding with viable prostate cancer cells (31). It is

postulated that 7E11 binds predominantly to apoptotic cells within prostate cancer sites *in vivo*. Apoptotic cells, unfortunately, comprise only a minority of the total prostate tumor-cell population. This, no doubt, has contributed to the relatively low sensitivity of <sup>111</sup>In-capromab pendetide as an imaging agent for prostate cancer. In this regard, targeting the extracellular PSMA domain with radioimmunoconjugates may enhance prostate cancer cell labeling *in vivo*.

The results of several but not all immunohistochemical studies using the 7E11 mAb have shown that PSMA is expressed in a limited number of nonprostatic tissues (1, 6, 15). Our findings support the results of other studies showing PSMA expression in duodenal (brush border) epithelium and renal proximal tubular epithelium but suggest that PSMA expression in these tissues is less than it is in prostate cancer and tumor-associated neovascularity (15, 16). Duodenal brush-border epithelium has high levels of folate hydrolase activity that is essential for folate absorption (17). This folate hydrolase activity is localized to the luminal membrane and is consistent with the staining pattern of the anti-PSMA mAbs. Proximal renal tubular epithelium also actively reabsorbs folate through the luminal membrane (32). Halsted *et al.* (33) found significant sequence homology between pig intestinal folate hydrolase (folypoly-gamma-glutamate carboxypeptidase) and human PSMA, suggesting that human duodenal membrane folate hydrolase may represent PSMA. Alternatively, it may represent a closely related enzyme that cross-reacts with anti-PSMA mAbs. In contrast to previous studies, we found consistent PSMA expression in mammary ductal epithelium. The reasons for our conflicting results are unclear; however, previous studies showing no PSMA expression in breast may have included specimens with inadequate amounts of ductal epithelium. One of our 12 colon specimens displayed PSMA expression in ganglion cells. The relatively sparse immunoreactivity observed in colonic ganglia may be indicative of peripheral neuronal PSMA expression previously described in non-myelinating, perisynaptic Schwann cells near motoneuron terminal endplates (34).

The staining profile of skeletal muscle is unique, in that a subset of cells is positive with only 7E11. Liu *et al.* (16) also showed a subset of skeletal muscle cells bind 7E11 and not other anti-PSMA mAbs. Of

Table 2 Results of PSMA immunohistochemistry using five different anti-PSMA mAbs in fresh-frozen benign tissue

Tissue	No. of positive cases/total no. of cases studied				
	7E11	J591	J415	PEQ226.5	PM2J004.5
Prostate	28/28	28/28	28/28	28/28	28/28
Lung	0/5	0/5	0/5	0/5	0/5
Brain	0/3	0/3	0/3	0/3	0/3
Digestive system					
Parotid	0/5	0/5	0/5	0/5	0/5
Esophagus	0/4	0/4	0/4	0/4	0/4
Stomach	0/6	0/6	0/6	0/6	0/6
Duodenum	11/11	11/11	11/11	11/11	11/11
Ileum	0/2	0/2	0/2	0/2	0/2
Colon	1/12	1/12	1/12	1/12	1/12
Pancreas	0/7	0/7	0/7	0/7	0/7
Liver	0/5	0/5	0/5	0/5	0/5
Genitourinary system					
Kidney					
Glomeruli	0/5	0/5	0/5	0/5	0/5
Proximal tubules	5/5	5/5	5/5	5/5	5/5
Distal tubules	0/5	0/5	0/5	0/5	0/5
Collecting ducts	0/5	0/5	0/5	0/5	0/5
Bladder	0/5	0/5	0/5	0/5	0/5
Testis	0/9	0/9	0/9	0/9	0/9
Breast	8/8	8/8	8/8	8/8	8/8
Ovary	0/5	0/5	0/5	0/5	0/5
Skin	0/5	0/5	0/5	0/5	0/5
Skeletal muscle	7/7	0/7	0/7	0/7	0/7
Endocrine system					
Thyroid	0/5	0/5	0/5	0/5	0/5
Adrenal cortex/medulla	0/5	0/5	0/5	0/5	0/5



Fig. 4. Skeletal muscle. A, H&E-stained section. B, 7E11 immunohistochemical stain showing positive reaction in a subset of cells. C, PM2J004.5 immunohistochemical stain showing no reactivity.

note is the fact that the other internal domain-binding anti-PSMA mAb, PM2J004.5, did not bind skeletal muscle. Thus, it is likely that, in skeletal muscle, 7E11 uniquely cross-reacts with either a yet to be defined PSMA-like or a PSMA-unrelated molecule. The patchy distribution suggests that expression of this molecule may be restricted to either fast-twitch or slow-twitch muscle fibers.

Novel PSMA-based prostate cancer therapies, including anti-PSMA mAb-based therapies, are currently under investigation (35–37). The results of our study indicate that anti-PSMA mAb-based diagnostic and therapeutic modalities may be expanded to include antineovascularity targeting for a wide variety of malignant neo-

plasms. The importance of angiogenesis in neoplasia is well documented (38–40), and endothelial cell expression of PSMA appears highly restricted to tumor-associated neovascularity and may represent a novel target for antineovascularity based therapy. Recent *in vivo* localization by the <sup>111</sup>In-labeled 7E11 mAb to a conventional (clear cell) renal cell carcinoma demonstrates the potential clinical utility of anti-PSMA mAbs in a nonprostate cancer (41). Enthusiasm for mAb-based therapy, however, must be tempered by the fact that PSMA is expressed in several benign tissue types; the potential side effects of anti-PSMA mAbs on these tissues *in vivo* is unknown. However, other mAbs that are currently in clinical trials or Food and Drug Administration-approved for clinical use, also are not tumor specific and bind antigens expressed in benign tissues (42, 43).

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